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The Implications of DNA Methylation for Toxicology: Toward Toxicomethylomics, the Toxicology of DNA Methylation

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Identifying agents that have long-term deleterious impact on health but exhibit no immediate toxicity is of prime importance. It is well established that long-term toxicity of chemicals could be caused by their ability to generate changes in the DNA sequence through the process of mutagenesis. Several assays including the Ames test and its different modifications were developed to assess the mutagenic potential of chemicals (Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D. (1973a). Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. U.S.A. 70, 2281–2285; Ames, B. N., Lee, F. D., and Durston, W. E. (1973b). An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. U.S.A. 70, 782–786). These tests have also been employed for assessing the carcinogenic potential of compounds. However, the DNA molecule contains within its chemical structure two layers of information. The DNA sequence that bears the ancestral genetic information and the pattern of distribution of covalently bound methyl groups on cytosines in DNA. DNA methylation patterns are generated by an innate program during gestation but are attuned to the environment *in utero* and throughout life including physical and social exposures. DNA function and health could be stably altered by exposure to environmental agents without changing the sequence, just by changing the state of DNA methylation. Our current screening tests do not detect agents that have long-range impact on the phenotype without altering the genotype. The realization that long-range damage could be caused without changing the DNA sequence has important implications on the way we assess the safety of chemicals, drugs, and food and broadens the scope of definition of toxic agents.

Key Words: **epigenetics; DNA methylation; gene expression.**

DNA METHYLATION PATTERNS AND EPIGENETIC HERITABILITY

The term "epigenetics" was coined by Waddington as a fusion of two older concepts that were at the center of ongoing controversy in embryology since the 17th century: "preformation" and "epigenesis." The question was whether the embryo was already preformed in the egg and that gestation expanded these preformed entities or whether the embryo went through a process of progressive steps of development producing something new and different from the egg in a process termed epigenesis. Waddington who recognized the role of genes in development fused epigenesis and "genetics" into the new term of epigenetics that referred to interaction of genes and "other" yet unknown factors in the process of development (Van Speybroeck, 2002). The realization that the same genes are present in all tissues in multicellular organisms but each cell type expresses different phenotypes brought forth the question of the relationship between genotype and phenotype that is cardinal for our discussion. It is interesting and entertaining from our perspective to note that originally it was thought that evolutionary principles of mutation and selection might explain development, embryogenesis was believed to involve a sequence of selected terminal genetic changes including mutation and gene amplification. The epigenetic concept introduced by Waddington provided a possible explanation for how one genotype could express multiple phenotypes without having to invoke a genetic change; genes go through unknown interactions that differentiate their functions and hence the phenotype in different cell types during development (Waddington, 1959, 1969). Waddington introduced two important concepts "canalization," which allows cells with identical genomes to take diverse trajectories and the "epigenetic landscape" that is formed through this process (Van Speybroeck, 2002). This original model has influenced our understanding of epigenetics as innate processes that result in canalized terminal differentiation that to a large extent is irreversible.

The concept of epigenetics implies that nongenetic events could generate stable phenotypic differences. Therefore, toxic

agents that are not mutagenic could cause stable adverse phenotypic changes if they interfered with epigenetic processes. The classic understanding that epigenetic processes are exclusively involved in embryonal development as proposed by Waddington would imply that toxic agents that interfered with epigenetic mechanisms would affect the phenotype only during gestation. One of the most elegant illustrations that interference of nongenotoxic agents in epigenetic processes during gestation would result in stable phenotypic changes was the demonstration in the agouti (A(vy)) mouse model that maternal dietary methyl content supplementation affected the coat color of her offspring through DNA methylation changes (Dolinoy *et al*., 2006, 2007a; Jirtle and Skinner, 2007; Waterland and Jirtle, 2003). This study provided evidence that a phenotype of an organism could be stably changed by exposure to a nongenotoxic agent during gestation.

However, the cardinal question is how terminal canalization is or how "high" are the walls of the canals that delineate the epigenetic landscape. Do epigenetic processes play a role in altering and modifying the phenotype beyond embryonal development? If indeed the epigenetic landscape is reversible after birth, then it is possible that toxic epigenetic agents influence the phenotype not only during gestation but also later in life.

The mysterious epigenetic processes proposed by Waddington are now understood in biochemical terms and allow new perspective on how nongenotoxic agents could trigger adverse phenotypic changes. There are several epigenetic mechanisms that are intensively investigated, and these include chromatin structure and histone modification that gate the access of transcriptional machinery to genes (Jenuwein and Allis, 2001; Strahl and Allis, 2000), noncoding RNAs including microRNA that regulates gene expression through altering chromatin configuration, inhibition of translation, and degradation of RNA (Bergmann and Lane, 2003), and remarkably, the DNA molecule itself bears epigenetic information encoded in the DNA methylation pattern (Razin and Riggs, 1980; Razin and Szyf, 1984), which will be the focus of our discussion.

DNA methylation is a covalent modification of DNA by addition of methyl residues to cytosine or adenine bases in DNA (Hotchkiss, 1948; Wyatt, 1950). DNA methylation in all organisms targets specific sequences. In vertebrates, the CG dinucleotide sequence is a principal target of DNA methylation (Gruenbaum *et al*., 1981) because it is preferentially recognized by vertebrate DNA methyltransferases (DNMT) (Gruenbaum *et al*., 1982). CG is the only dinucleotide sequence that contains a cytosine that is a palindrome and could be copied during cell division by a semiconservative DNMT from the parental strand onto the daughter strand (Razin and Riggs, 1980). Thus, changes introduced into the DNA methylation pattern either stochastically or as an organized response to developmental or environmental signals could be maintained and memorized through DNA replication cycles. This is a mechanism through which a transient exposure to an environmental agent could result in lasting impact on DNA methylation and as a consequence on the phenotype. However, recent data including genomic sequencing suggest that DNA methylation occurs in other dinucleotide sequences in addition to CG in undifferentiated cells (Fuso *et al*., 2010; Lister *et al*., 2009). It remains to be seen, however, whether non-CG methylation is present, albeit at lower level, in other differentiated tissues such as the brain and whether it plays a role in dynamic DNA methylation responses throughout life. The presence of non-CG methylation in the genome suggests that at least certain methylation marks are not automatically mitotically heritable as predicted by the classical model of semiconservative mitotic heritability of DNA methylation as will be discussed below and are maintained dynamically by a balance of methylating and demethylating enzymes. However, DNA methylation changes in both CG and non-CG sites could potentially mediate the long-term impact of exposure to environmental agents, although there might be different mechanisms of maintenance of these different methylated sequences.

What distinguishes DNA methylation in vertebrates is that not all potential methylatable sequences are methylated in all cells in a given individual. The same site might be methylated in several cell types but not others. This creates a cell type–specific pattern of methylation. Cell type–specific patterns of methylation or tissue-specific differentially methylated regions were discovered in the early 80's using methylation-sensitive restriction enzymes (Benvenisty *et al*., 1985; Razin and Szyf, 1984) and were confirmed three decades later by high-throughput genomic sequencing (Lister *et al*., 2009). Thus, in addition to the individual identity that is encoded in the sequence of the four bases in DNA, there is a cell type identity encoded in the distribution of methyl moieties in the same molecule of DNA. Cell type–specific differentially methylated regions provide an elegant explanation for the question of how could the same genome encodes multiple stable phenotypes in a multicellular organism. DNA methylation provides cell type identity to genomes, identical DNA sequence could bear different DNA methylation patterns in different cell types. Thus, alternations in DNA methylation triggered by a toxic environmental agent could have tissue-specific manifestation. This adds a level of complexity to the screening for DNA methylation–modifying agents as the agents might have a diverse impact on the DNA methylation pattern and the gene expression profile in different tissues.

POSSIBLE ROLES OF DNA METHYLATION: CONTROL OF GENE EXPRESSION THROUGH PROMOTER METHYLATION

The role of DNA methylation should be understood within the broader context of chromatin structure. DNA methylation patterns in vertebrates are distinguished by their overall correlation with chromatin structure. Active regions of the chromatin, which enable gene expression, are associated with

hypomethylated DNA, whereas hypermethylated DNA is packaged in inactive chromatin (Razin and Cedar, 1977). This link between active chromatin and hypomethylation points to the possibility that DNA methylation might be regulating differential gene expression.

It has been known for more than two decades that DNA methylation in regulatory regions such as promoters and enhancers could silence gene expression, and an inverse correlation between gene expression and DNA methylation in promoters was proposed (Razin and Szyf, 1984). Recent whole-genome approaches revealed that promoters of vertebrate genes were generally devoid of DNA methylation and that overall promoter DNA methylation and gene expression were inversely correlated (Rauch *et al*., 2009). However, it is clear that a large fraction of promoters that are unmethylated do not show an obvious correlation with differential gene expression. Silencing of these genes in certain tissues can occur without clear hypermethylation. It is important in this context to differentiate between measurements of gene expression that define a transient state of expression and DNA methylation states that measure the programming and conditioning of a gene for expression. A gene might be conditioned for expression by hypomethylation but will be only expressed in response to specific triggers such as hormones. It is possible that some of the inconsistencies in the relationship between differential DNA methylation and differential gene expression could be derived from these differences between transient states of expression and a programmed conditioning for expression. A more trivial explanation is that not all regulatory regions of genes are known and that other DNA methylation–dependent regulatory regions exist that could explain the discrepancies between gene expression and DNA methylation.

Methylation of CGs in promoters is an extremely effective mechanism of silencing expression in expression vector assays in tissue culture (Stein *et al*., 1982) almost with no exception. Two important mechanisms for inhibition of gene expression by promoter DNA methylation are well established. First, methyl cytosines in the recognition elements of transcription factors could block their binding resulting in reduced transcriptional activity (Comb and Goodman, 1990; Inamdar *et al*., 1991). A second mechanism involves recruitment of methylated DNA–binding domain proteins (MBD) to methylated cytosines in promoters (Nan *et al*., 1997). MBDs recruit histone-modifying complexes containing histone deacetylases (HDACs) such as the NurD complex and histone methyltransferases (HMTase) to promoters resulting in an inactive chromatin configuration around the genes (Eden *et al*., 1998).

The involvement of chromatin modification enzymes in the mechanism of gene silencing by DNA methylation points to possible synergistic interactions between DNA methylation and chromatin structure. Although it was originally believed that the relationship between DNA methylation and chromatin inactivation was unilateral and DNA methylation precipitated an inactive chromatin structure (Eden *et al*., 1998; Nan *et al*., 1997), data accumulated in the last 10 years point to a bilateral relationship between DNA methylation and chromatin modification. For example, K27 methylation and the HMTase EZH2 target DNMT to specific sites in the genome (Vire *et al*., 2006). On the other hand, increasing histone acetylation can cause DNA demethylation (Cervoni and Szyf, 2001; Selker, 1998). This bilateral relationship between chromatin and DNA methylation (D'Alessio and Szyf, 2006) creates a possible conduit for altering DNA methylation in a more dynamic way than previously thought because chromatin modification is dynamic. This is especially important in the brain. It also creates a link between agents that change chromatin modifications and DNA methylation aberrations.

The bilateral relationship between DNA methylation and chromatin modifications has important implication in toxicology. Agents that do not affect the DNA methylating/demethylating enzymes *per se* might still affect DNA methylation through inhibiting chromatin-modifying enzymes. Thus, such agents could have a long-term impact on the DNA methylation pattern and as a consequence the phenotype. An excellent example is valproic acid, a drug that has been used for decades as a mood stabilizer and antiepileptic drug. Valproic acid also inhibits HDAC and increases histone acetylation (Gottlicher *et al*., 2001) and at the same time stimulates DNA demethylation probably through facilitating access of regulatory regions of genes to demethylases (Detich *et al*., 2003a; Ou *et al*., 2007).

DIFFERENTIAL DNA METHYLATION REGIONS OUTSIDE PROMOTERS

Differentially methylated regions are not limited to promoters, suggesting that DNA methylation might play a role in controlling genome function beyond promoter/enhancer regulation of gene expression. Our recent unpublished data suggest that DNA methylation differences that associate with early-life adversity include intergenic regions as well as gene deserts (McGowan *et al*., 2011). Global changes in DNA methylation are common in several chronic diseases such as cancer (Feinberg and Vogelstein, 1983) and lupus (Cornacchia *et al*., 1988; Yung and Richardson, 1994), and these must include broad genomic regions well beyond promoters and enhancers. Although it is possible to brush off these changes as an epiphenomenon, the consistency of global hypomethylation in cancer and other diseases suggests that there are additional modalities through which DNA methylation exerts its impact on the genome. Indeed, measuring global DNA methylation levels is potentially an important test to screen deleterious agents. However, global methylation assays define an average methylation, and important changes involving increases in some regions of the genome coupled with decreases in other regions might not be detected if one simply looks at the average level of methylation.

One possible role for ''global methylation'' that has been supported by experimental and genetic evidence is that it is responsible for structural organization of the genome and that global loss of DNA methylation could result in genomic instability. For example, immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF), a rare chromosome breakage disease that involves ICF, is characterized by global hypomethylation and is caused by a mutation in the DNMT3B (Ehrlich, 2003; Hansen *et al*., 1999; Okano *et al*., 1999). Murine embryonic stem cells nullizygous for the DNMT1 exhibit increased mutations and chromosomal aberrations (Chen *et al*., 1998). It has been well known that hypomethylating agents cause widespread chromosomal rearrangements (Ji *et al*., 1997). An alternative explanation is that these global changes in DNA methylation play a role in a higher order control of gene expression and genome function. It is especially becoming clear now that genes act in interacting networks that involve multiple genes as well as higher order networks. Global changes in DNA methylation might be playing at this level and could possibly serve as adaptive responses to reorganize and reset higher order genome function. We will discuss this below in the context of epigenomic changes driven by the environmental exposures.

Although most of the attention in the field has focused on promoter DNA methylation, recent data suggest that gene-body DNA methylation positively correlates with gene expression in plants (Zhang *et al*., 1999) and vertebrates (Hellman and Chess, 2007; Lister *et al*., 2009; Rauch *et al*., 2009). A revealing example is X inactivation in females; although promoters in the active X are unmethylated relative to the inactive X, the reverse is true for the bodies of genes that are hypermethylated on the active X (Hellman and Chess, 2007). It is interesting to note that high-density mapping of genome-wide DNA methylation by deep sequencing of bisulfite DNA has revealed that once we move away from the promoter region, the simple notion of an inverse correlation between methylation and expression falls apart (Boellmann *et al*., 2010).

It is unclear yet how gene-body methylation might be involved in regulating gene expression. It is possible that DNA methylation in gene bodies suppresses spurious firing of cryptic promoters including antisense promoters, thus facilitating firing from the correct start site. Indeed, a recent paper has shown that gene-body DNA demethylation causes reduced expression and processing of ribosomal RNA genes by allowing cryptic RNApolII firing (Gagnon-Kugler *et al*., 2009). It should be noted that gene-body methylation might play a role in promoter regulation by controlling the expression of alternative downstream promoters (Maunakea *et al*., 2010).

The state of methylation of body of genes and intergenic regions might be of biological relevance. It is anticipated that with the emergence of whole-genome technologies that allow examination of differentially methylated regions beyond promoter and CG islands, the scope of DNA methylation involvement in gene and genome function will be broadened. This is especially important for interpretation of studies that are attempting to link interindividual differences in environmental exposures and interindividual differences in DNA methylation. Our preliminary data indicate that a large fraction of differences in DNA methylation appear in regions beyond traditional promoter boundaries (McGowan *et al*., 2011). The possibility that DNA methylation changes outside canonical promoter regulatory regions might have adverse biological effects should impact any future assay developed to detect DNA methylation–modifying agents. Whole-genome methods that cover intergenic sequences using a combination of methylated DNA immunoprecipitation with high-density microarrays or genome-wide deep sequencing of bisulfite-converted DNA could be employed (Boellmann *et al*., 2010). Focusing on promoters alone might result in misclassification of agents that might have an impact on genome function.

In summary, the well-established role of DNA methylation has been programming gene expression through promoter/enhancer methylation during gestation and embryonal development conferring cellular identity (Razin and Riggs, 1980), regulating X inactivation (Riggs, 1975), parental imprinting (Sapienza, 1990), and silencing parasitic elements in the genome (Bestor, 1998). Recent analysis of differentially methylated regions suggests other modalities for DNA methylation in controlling genome function as well as a broader scope for the biological involvement for DNA methylation. Agents that affect DNA methylation might alter not only promoter function but also other processes regulated by DNA methylation such as silencing of spurious transcription initiated at the wrong positions in bodies of genes or in the antisense orientation, genome structure, and noncoding RNA transcription.

DNA METHYLATION PATTERNS AND THE IMPACT OF TOXIC AGENTS DURING EMBRYOGENESIS

Differential methylation patterns are generated during gestation by DNA methylation and demethylation enzymatic activities, and these patterns are maintained thereafter to guard the stability of the differentiated cell phenotype (Razin and Riggs, 1980). Cellular differentiation of pluripotent cells involves extensive changes in DNA methylation (Razin *et al*., 1984). Either the DNA methylation inhibitor 5-azaC (Jones and Taylor, 1980) or depletion of DNMT by antisense knockdown triggers cellular differentiation (Szyf *et al*., 1992). As expected, recent high-throughput sequencing of the DNA methylome confirms that stem cell differentiation is associated with extensive changes in methylation of differentially methylated regions (Lister *et al*., 2009).

Embryogenesis is an extremely critical point in life when the DNA methylation pattern is susceptible to possible disruption by environmental exposures. Agents that interfere with DNA methylation enzymes during the critical time when these patterns are generated could disrupt the DNA methylation

pattern and disturb cellular differentiation and organogenesis. DNA methylation alterations could target different regions of the genome and would range from effects that disrupt embryogenesis and cause overt teratogenesis to changes that become noticed only later in life. It has been postulated that some late-onset adult diseases are caused by DNA methylation alterations early in life (Dolinoy and Jirtle, 2008; Gluckman *et al*., 2009a; Hanson and Gluckman, 2008; Sinclair *et al*., 2007b). Teratogenic events are obvious and relatively easy to detect in animal models. However, a critical challenge is to develop methods to screen and detect agents that interfere with DNA methylation during gestation whose phenotypic effect is latent and might manifest itself only later in life. The dramatic increase in frequency of chronic adult diseases including type II diabetes and autoimmune diseases point to the urgency of addressing this question. Moreover, several of the known teratogens such as valproic acid might have latent effects as well, even when there are no noticeable overt effects at birth. "Normal" neonates born to valproic acid–exposed mothers might bear methylation alterations that would result in disease or behavioral pathologies later in life. Identifying these subjects at risk is important. This issue to my opinion has not been properly addressed.

There are well-documented examples of environmental exposures that interfere with DNA methylation during embryogenesis. Examples are nutritional restriction during pregnancy (Unterberger *et al*., 2009) and low-folic acid/vitamin B12 diets during the periconception period (Sinclair *et al*., 2007a). Reducing the level of methyl donors in cells could result in inhibition of DNA methylation and facilitation of demethylation activity. Increased methyl content in the diet on the other hand would result in increased DNA methylation activity and reduced DNA demethylation (Detich *et al*., 2003b). Another documented example is bisphenol A (BPA), a high production volume chemical used in the manufacture of polycarbonate plastic, which causes DNA demethylation. Exposure *in utero* to this agent is associated with higher body weight, increased breast and prostate cancer, and altered reproductive function (Ho *et al*., 2006). On the other hand, gynestein found in soybean could cause an increase in DNA methylation and counteract the effects of BPA (Dolinoy *et al*., 2007b).

It is also well documented that agents that interfere with DNA methylation during embryogenesis could have long-term life-long impact on the phenotype that is not teratogenic *per se*. Nutritionally induced epigenetic changes *in utero* are likewise linked with chronic disease formation in adulthood. For example, obesity might be an adaptive response to famine that is anticipated by nutritional restriction *in utero*. Indeed, it is well documented that DNA methylation changes occur in response to nutritional restriction during gestation (Ke *et al*., 2006; MacLennan *et al*., 2004; Sinclair *et al*., 2007a). Yet unknown adaptive programs that sense the nutritional state and respond to it by programming alterations in epigenetic patterns might mediate the effect of nutritional restriction during gestation. Diet components might also directly affect the cellular level of methyl donors during gestation. This can result in either increased or decreased DNA methylation of critical genes during critical times in development when DNA methylation patterns are formed. Folic acid deficiency during gestation results in an array of teratogenic effects, which might be explained by reduced methyl availability in cells and reduced supply of *S*-adenosyl methionine (SAM) driving the DNA methylation reaction (Brunaud *et al*., 2003; Pogribny *et al*., 1997; Wainfan *et al*., 1989).

The strongest evidence for the effect of maternal diet on the phenotype of the offspring comes from an elegant experiment done by the Jirtle group. The *agouti* locus controls the distribution of yellow and black hair pigments. Mice bearing the agouti viable yellow allele (A(vy)) harbor a transposable element in the agouti gene. Waterland and Jirtle (2003) demonstrated that methyl supplementation of dams bearing the nonagouti alleles a/a dams with extra folic acid, vitamin B (12), choline, and betaine altered the phenotype of their A(vy)/a heterozygous offspring via increased CpG methylation at the transposable element of A(vy) locus. Using the same model, these researchers later showed that maternal supplementation of Genistein, the major phytoestrogen in soy, during gestation, at levels comparable with humans consuming high-soy diets, shifted the coat color of heterozygous viable yellow agouti (A(vy/a) offspring toward pseudoagouti. This was significantly associated with increased methylation in a retrotransposon upstream of the transcription start site of the *Agouti* gene. This genistein-induced hypermethylation persisted into adulthood, decreasing ectopic *Agouti* expression and protecting offspring from obesity. This was the first evidence that *in utero* dietary genistein affects gene expression through DNA methylation and thus alters susceptibility to obesity in adulthood by altering the epigenome (Dolinoy *et al*., 2006).

A recent study provided the first evidence in humans that nutritional restriction during gestation resulted in life-long alterations in DNA methylation patterns. The Dutch Hunger Winter of 1944–1945 and its long-term effects on health of humans who were exposed to these conditions prenatally are well documented and were long considered to be a prime candidate example for epigenetic programming by gestational nutritional restriction. Individuals who were prenatally exposed to this famine had, six decades later, less DNA methylation of the imprinted *IGF2* gene compared with their unexposed, same-sex siblings. However, the DNA methylation differences between the groups were small and were detected only using age-adjusted linear mixed models. An association was found only for periconceptional exposure, consistent with the hypothesis that very early mammalian development is a crucial period for establishing and maintaining epigenetic marks (Heijmans *et al*., 2008).

Agents that could cause a change in DNA methylation might not act on the DNA methylation enzymes but on the chromatin

modification enzymes. For example, as discussed above, valproic acid a commonly used drug that is also a teratogen is an HDAC inhibitor. Others and we have shown that valproic acid could cause demethylation of DNA (Alonso-Aperte *et al*., 1999; Detich *et al*., 2003a; Dong *et al*., 2007, 2008; Milutinovic *et al*., 2007; Zhang *et al*., 2004). Although it is not certain what is the mechanism responsible for the teratogenicity of valproic acid, it stands to reason that it is caused by DNA demethylation. Valproic acid causes neural tube defects similar to methyl-deficient diets, and its biological effect could be inhibited by a methyl-rich diet (Ornoy, 2009), supporting the hypothesis that its mechanism of toxicity involves loss of DNA demethylation. A screen for agents that interfere with DNA methylation should also consider the possibility that they might act indirectly through affecting chromatin modification.

Although the common wisdom is that agents that interfere with DNA methylation machinery and inhibit general DNA methylation enzymes during embryogenesis will cause stochastic changes in DNA methylation, an alternative hypothesis is that changes in DNA methylation in response to different agents might be an adaptive and organized response that is system wide and genome wide. The scope of exposures that might lead to genome-wide DNA methylation adaptations might be broader than the range of chemicals that interfere with DNA methylation/demethylation enzymes. If this is the case, an adaptive response to an environmental agent that involves changes in DNA methylation might be triggered by an array of agents that do not target DNA methylation enzymes *per se*. This possibility calls for developing screens to identify exposures that elicit either an adaptive or stochastic DNA methylation response during gestation.

ENZYMES INVOLVED IN THE DNA METHYLATION REACTION

The DNA methylation pattern is generated and maintained by enzymes. This is different from the DNA sequence, which is replicated by the DNA replication complex, but the identity of the sequence that is replicated is exclusively dependent on the template. Therefore, although it is possible to block DNA replication by agents that inhibit DNA replication enzymes, it is impossible to change the sequence in a predictable way by such agents except through mutagenesis. The DNA methylation pattern is generated by enzymatic reactions that are independent of Watson and Crick rules. Blocking DNA methylating enzymes during DNA replication, e.g., would alter the DNA methylation pattern (Jones and Taylor, 1980). Therefore, different agents that interfere with DNA methylation enzymes and other partners of the DNA methylation machinery can alter DNA methylation patterns (Fig. 1). This has therapeutic and toxic implications. Agents that interfere with DNA methylation processes could be used to remove deleterious DNA methylation therapeutically or on the other hand could introduce DNA methylation aberration with toxic consequences. Understanding the scope of the biological processes controlled by DNA methylation that could be disrupted by potentially toxic agents is guided by an appreciation of the mechanism responsible for generating and maintaining DNA methylation patterns. The main question is whether DNA methylation patterns are labile only during gestation as discussed above or whether they would be labile throughout life as well. An additionally critical issue is whether DNA methylation is vulnerable to modification exclusively in dividing cells or could it be modified in nondividing cells such as neurons or heart muscle as well. Addressing these questions is essential for delineating the scope of effects that agents that interfere with DNA methylation might have on health. For example, are adults protected from the deleterious effects of agents that modify DNA methylation? Are there critical periods in life? Would agents that block DNA methylation change DNA methylation patterns in the brain and heart as well? This has become especially important recently because DNA methylation inhibitors were introduced into clinical practice, but obviously this concern is not limited to these agents. The type of screens that will be used to identify agents that alter DNA methylation will have to take these questions into account.

It has been widely accepted that the main biological role of DNA methylation is to participate in establishment of differentiated cell types during embryogenesis. If DNA methylation is indeed a guardian of an innate developmental program of cell fate differentiation, it must be kept faithfully after birth and throughout life and it must be consistent across individuals. In this case, the enzymatic activity would be geared toward guarding the DNA methylation pattern after birth without change. Our understanding that DNA methylation served as a heritable epigenetic mechanism differentiating cellular phenotypes during embryogenesis drove our classical ''first principles'' of DNA methylation. The most compelling concept in this respect is the ''semiconservative'' process of copying DNA methylation patterns, the ability of a methylated cytosine in CG to accurately replicate its methylation pattern during mitosis onto a daughter strand of DNA (Gruenbaum *et al*., 1982) (Fig. 1). CG DNA methylation is thus uniquely positioned to bear the epigenetic code. There is no other epigenetic mark that could be automatically replicated like methylCG. The other important element of this idea is that there is no *de novo* methylation or demethylation occurring in differentiated cells. Such activities could potentially disrupt the integrity of DNA methylation in differentiated cells. This model predicts that DNA methylation could be changed only during cell division. Therefore, toxicity caused by DNA methylation interfering agents would be limited to gestation and after birth to dividing cells. In such cells, only inhibition of maintenance of DNMT during cell division would result in synthesis of DNA that is unmethylated, but there will be no effect of adding DNA methylation by *de novo* methylation in

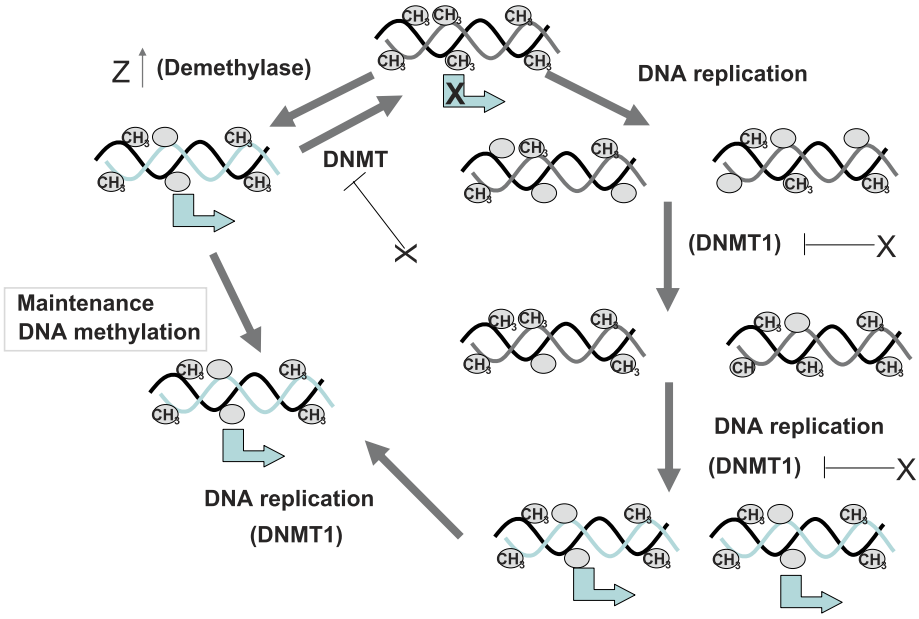

FIG. 1. Toxic agents affecting DNA methylation in dividing and nondividing cells, a model. In dividing cells, inhibition of DNMT1, the maintenance of DNMT, by putative toxic agent X during DNA replication will result in loss of methylation (CH_3) from certain sites. If the agent X is present in the next round of replication, both strands of DNA are demethylated at this position and a gene that was silenced by the methylated regulatory element is activated (indicated by the horizontal arrow). Once the site of methylation is lost on both strands, the situation is maintained for further cell divisions in the absence of the toxic agent X. The state of methylation acts as a memory in the genome to the transient exposure to substance X. The left side of the diagram shows the situation in a postmitotic cell. The balance of DNA methylation is defined by an equilibrium of methylating enzyme DNMT and demethylases. Putative toxic substance Y induces demethylase activity resulting in demethylation of a regulatory region and activation of transcription. Alternatively, substance X inhibits DNMT, tilting the balance between DNMTs and demethylases toward demethylase resulting in demethylation and activation of the gene (horizontal arrow). The new state of methylation is long-term maintained by the balance of DNMT and demethylases in the absence of the toxic agents.

all cases (Fig. 1). This concept has dominated our thinking in the area of DNA methylation pharmacology and toxicology.

However, if on the other hand DNA methylation has any role in modulating genome function after birth and if DNA methylation is responsive to extracellular environmental agents during postnatal development, childhood and adolescence, it must remain dynamic after birth and the presence of methylation and demethylation enzymes should allow for a dynamic balance to be maintained. This must therefore include enzymes that add DNA methylation and those that remove DNA methylation. These enzymes might be vulnerable to candidate toxic agents throughout life. Toxicity of DNA methylation–modifying agents would in this case include postmitotic tissue and possibly neurons (Fig. 1).

DNA Methyltransferases

The DNA methylation reaction is catalyzed by DNMT (Razin and Cedar, 1977). Methylation of DNA occurs immediately after replication by a transfer of a methyl moiety from the donor SAM (AdoMet) in a reaction catalyzed by DNMT. Three distinct phylogenic DNMT were identified in mammals. DNMT1 shows preference for hemimethylated DNA *in vitro*, which is consistent with its role as maintenance of DNMT, whereas DNMT3a and DNMT3b methylate unmethylated and methylated DNA at an equal rate, which is consistent with a *de novo* DNMT role (Okano *et al.*, 1998). Two additional DNMT homologs were found: DNMT2 whose substrate and DNA methylation activity is unclear (Vilain *et al.*, 1998) but was shown to methylate transfer RNA (Goll *et al.*, 2006; Rai *et al.*, 2007) and DNMT3L, which lacks key methyltransferase motifs, is a regulator of DNMT3a and DNMT3b and is essential for the establishment of maternal genomic imprints and *de novo* methylation of retrotransposons (Bourc'his *et al.*, 2001, Bourc'his and Bestor, 2004). Knockout mouse data indicate that DNMT1 is responsible for a majority of DNA methylation marks in the mouse genome (Li *et al.*, 1992) as well as the human genome (Chen *et al.*, 2007), whereas DNMT3a and DNMT3b are responsible for some but not all *de novo* methylation during development (Okano *et al.*, 1999).

Razin and Riggs (1980) proposed that the DNA methylation pattern was accurately and automatically inherited during replication because maintenance of DNMT could only methylate hemimethylated sites. Hemimethylated sites are generated on the nascent DNA strand during DNA replication when a methylated CG dinucleotide in the template strand is replicated (Fig. 1). DNA methylation was therefore proposed to be truly heritable by an automatic semiconservative mechanism similar to DNA replication (Razin and Riggs, 1980).

TABLE 1
Properties of Mammalian DNMT (Jeltsch, 2006).

Enzyme	Recognition sequence in DNA	Substrate preference	Comment
DNMT1	CG	Hemimethylated DNA	Maintenance of methyltransferase
DNMT2	?	tRNA	Unknown
DNMT3L			Regulator of DNMT3a and DNMT3b; no catalytic activity
DNMT3a	CG/non-CG	Hemimethylated and unmethylated	*De novo* methyltransferase
DNMT3b	CG/?	Hemimethylated and unmethylated	*De novo* methyltransferase

Note. The question mark implies that the recognition sequence in DNA is unknown.

It is becoming clear now, however, that DNMTs are targeted to specific sequences and that the targeting factors are required not only for generating the patterns of methylation but also for maintaining the pattern of DNA methylation. For example, it has recently been demonstrated that an additional factor, the protein ubiquitin-like, containing PHD and RING finger domains 1, also known as NP95 in mouse and ICBP90 in human, is required for targeting DNMT1 to newly replicating hemimethylated DNA (Bostick *et al*., 2007). Therefore, the outcome of DNMT inhibition by an environmental agent should be defined not only by its effect on the global activity of DNMTs but also by the scope of targeting factors and their binding to different genomic targets in different cell types.

Generation and maintenance of specific DNA methylation patterns requires an interaction with chromatin-modifying enzymes. An excellent example is the polycomb complex PRC1 and specifically the HMTase EZH2 (Vire *et al*., 2006). This complex guides DNMT1 and DNMT3A to specific sites in the genome. The presence of EZH2 is required not only for generation of DNA methylation sites but also for maintaining these sites methylated. Again supporting the idea that even in somatic cells, the DNA methylation pattern is not automatically copied and maintained. Therefore, agents that target EZH2 or agents that disrupt the interaction between DNMTs and the polycomb repressive complex 1 complex could cause changes in DNA methylation. This again has implications on how we design assays for toxicity of agents that disrupt DNA methylation.

DNMTs are found in complexes with other proteins that include other chromatin-modifying proteins such as HDAC1 and HDAC2 (Fuks *et al*., 2000, 2003; Rountree *et al*., 2000; Vire *et al*., 2006). The pharmacophore of diverse DNMT-containing complexes might be different, and these complexes would react differently with small molecule inhibitors than naked DNMT. Different environmental agents might exhibit selectivity to different complexes. Another unresolved issue is the extent of participation of the different DNMT isoforms in maintaining DNA methylation patterns and the identity of their unique targets in the genome (Chik and Szyf, 2010).

DNA Demethylases

The original thinking in the field was that DNA methylation pattern remained fixed in postmitotic cells because there was no activity that could remove methyl groups from DNA, the only way DNA methylation patterns could be altered was by inhibition of DNMT during DNA synthesis when new unmethylated strands of DNA are generated. This implied that only agents that blocked maintenance of DNA methylation during cell division could possibly affect the DNA methylation pattern in somatic cells. Cells that did not divide were considered to be immune from DNA methylation–modifying agents. If demethylases were present in nondividing cells, then there was the possibility that toxic agents could impact the DNA methylation pattern even in fully differentiated postmitotic cells (Figs. 1 and 2). This has profound implications for epigenetic toxicology because it expands the scope of adverse activities of candidate toxic agents that influence DNA methylation.

We have proposed in the past that an active replication-independent mechanism was responsible for global hypomethylation triggered by Epstein Barr virus viral replication-inducing agents such as butyric acid (Szyf *et al*., 1985), and we later proposed that DNA methylation is a reversible biological signal like other common biological signals (Bhattacharya *et al*., 1999; Ramchandani *et al*., 1999). If indeed demethylases are present in somatic cells, it implies that the DNA methylation pattern is preserved and does not drift to a demethylated state because there is a balance of methylation and demethylation activities. Inhibiting one side of the DNA methylation reaction by an interfering agent could tilt this balance. For example, in nondividing cells, the inhibition of DNA methylating enzymes would tilt the balance toward DNA demethylation, and the net result would be demethylation. In contrast, inhibiting demethylating enzymes would result in tilting the balance toward increased DNA methylation by DNA methylating enzymes (DNMTs) (Fig. 2).

The idea that DNA methylation could be reversed enzymatically (Ramchandani *et al*., 1999) has been extremely controversial. It is now, however, well accepted that active demethylation does occur (Bruniquel and Schwartz, 2003;

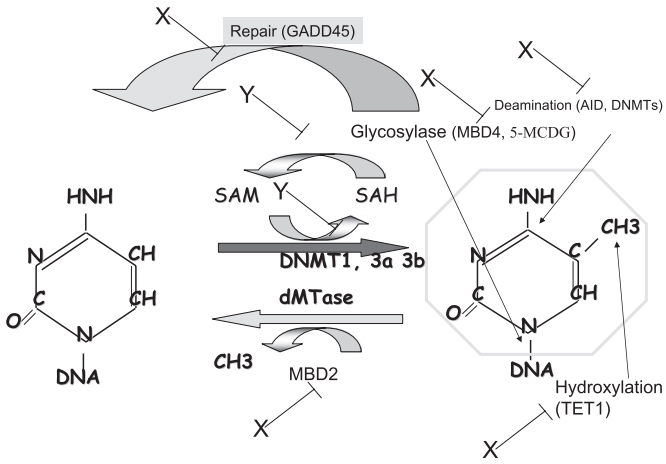

FIG. 2. The DNA methylation equilibrium; enzymatic targets of DNA methylation–modifying toxic agents. DNA is methylated by a transfer of a methyl moiety from the methyl donor SAM to the 5′ position on a cytosine ring by DNMT releasing S-adenosyl-homocysteine (SAH). SAM is regenerated by the following sequence of reactions: (1) hydrolysis of SAH to homocysteine by homocysteine hydrolase, (2) the methylation of homocysteine to methionine by methionine synthase, and (3) the adenylation of methionine to SAM by SAM synthetase. X-putative agents that could cause demethylation. Toxic agents (X) that inhibit either DNMTs or the four reactions involved in the synthesis of SAM will affect the DNA methylation equilibrium and reduce the drive toward DNA methylation, allowing an increase in DNA demethylation. Several demethylation reactions were suggested. Direct demethylation by a demethylase enzyme (dMTase) (MBD2 is a putative candidate) could release a methyl moiety (CH3) in the form of either methanol or formaldehyde. Alternatively, the methyl cytosine ring could be modified either by deamination catalyzed, e.g., by AID, or by the DNMT, which were shown to catalyze deamination of 5-methylcytidine in the absence of SAM or hydroxylation of the methyl moiety catalyzed by TET1. The modified base is then excised and repaired. Alternatively, the bond between the sugar and the base is cleaved (by glycosylases such as MBD4 or 5-methylcytosine glycosylase 5-MCDG) followed by repair. Repair proteins shown to be associated with demethylation were GADD45(a and b). Y-putative toxic agents that interfere with the different demethylation activities. These agents will reduce the drive toward demethylation and tilt the equilibrium to higher methylation activity resulting in hypermethylation.

Lucarelli *et al*., 2001; Oswald *et al*., 2000; Szyf *et al*., 1995; Wilks *et al*., 1984), and it has been shown that brain extracts are capable of demethylating "naked" DNA substrate (Dong *et al*., 2008; Mastronardi *et al*., 2007). The most remarkable observations illustrating a dynamic methylation-demethylation equilibrium in postmitotic cells come from the brain where several studies have already shown demethylation in postmitotic neurons (Feng and Fan, 2009, 2010; Levenson *et al*., 2006; Miller and Sweatt, 2007; Weaver *et al*., 2004). Accordingly, conditional knockout of DNMT1 in postmitotic neurons results in DNA demethylation (Feng and Fan, 2009).

Although there is agreement that demethylation happens, the main disagreement is on mechanism. Because there is a reluctance to accept that a methyl group could enzymatically be removed from a cytosine ring in DNA, the most widely accepted mechanisms for active DNA demethylation involve selective DNA repair that results in replacement of the methylated cytosine base by an unmethylated cytosine base (Fig. 2). One proposed mechanism is that the methylated cytosine base is cleaved from the deoxyribose by a glycosylase activity, the abasic site is then repaired and replaced with an unmethylated cytosine (Jost, 1993; Razin *et al*., 1986). Another proposal is that the initiator of the repair-based replacement of 5 methylcytosine is deamination of the 5 methylcytosine base to a thymidine creating a C/T mismatch that is corrected by a mismatch repair activity. Demethylation in zebrafish embryos was shown to involve a complex sequence of coupled enzymatic reactions; activation-induced (cytidine) deaminase, which converted 5-methylcytosine to thymine, a G:T mismatch-specific thymine glycosylase (MBD4), removed the thymidine, which was followed by repair promoted by GAD45 (Rai *et al*., 2008). AID has been implicated in the global demethylation in mouse primordial germ cells (Popp *et al*., 2010). Interestingly, bacterial DNMT *Hha*I was previously shown to catalyze deamination of 5 methylcytosine to thymidine under conditions where SAM (the methyl donor) was unavailable (Shen *et al*., 1992; Zingg *et al*., 1998). It was recently proposed that vertebrate DNMTs could also participate in demethylation by deaminating the methylcytosine to thymidine (Kangaspeska *et al*., 2008). If this is true, agents that target DNMTs might act as inhibitors of deamination-triggered DNA demethylation resulting in opposite consequences to the expected inhibition of DNA methylation. GADD45a, a DNA repair protein, was proposed to participate in catalysis of active DNA demethylation in mammals by an unknown DNA repair–based mechanism (Barreto *et al*., 2007); however, this was disputed (Jin *et al*., 2008).

In contrast to these repair-based mechanisms, we have previously proposed that demethylation is truly a reversible reaction that involves removal of the methyl moiety rather than breaking the DNA and fixing it with an unmethylated cytosine (Ramchandani *et al*., 1999). We proposed that the MBD2 was a *bona fide* demethylase that removed methyl groups from DNA and truly reversed the DNA methylation reaction. This is to date the only described *bona fide* demethylase. MBD2 has been implicated in the activation of both methylated and unmethylated genes (Angrisano *et al*., 2006; Fujita *et al*., 2003). Several groups (Ng *et al*., 1999; Wade *et al*., 1999) have contested the demethylase and transcriptional activating properties of MBD2. Studies by Detich *et al*. (2002) have demonstrated, however, MBD2 demethylase activity *in vitro*. Hamm *et al*. (2008) have proposed an oxidative mechanism of 5-methylcytosine DNA demethylation by MBD2. According to this mechanism, oxidation of the methyl moiety generates 5-hydroxymethylcytosine by oxidation, which is followed by release of the methyl residue in formaldehyde. Interestingly, 5-hydroxymethylcytosine was recently discovered in mammalian DNA (Kriaucionis and Heintz, 2009). A recent study has shown that ten-eleven translocation-1 (TET1), an enzyme that converts methylcytosine to hydroxymethylcytosine, is required for maintaining the demethylated state of *nanog* in embryonic

stem cells supporting a possible role for TET1 and 5-hydroxymethylcysoine as an intermediary in the demethylation reaction (Ito *et al*., 2010). In summary, although there is no agreement as of yet on the mechanism of DNA demethylation, the presence of active demethylation in somatic cells is widely acknowledged. It is probable that all the mechanisms that were proposed and were listed above are indeed involved in DNA demethylation under some conditions. This expands the scope of targets for agents that could alter the DNA methylation pattern by inhibiting demethylation.

INTERRELATIONSHIP BETWEEN CHROMATIN MODIFICATION AND DNA DEMETHYLATION: IMPLICATION FOR TOXICOLOGY

As discussed above, several lines of evidence indicate that chromatin modification states and DNA methylation states are interrelated, and therefore, it is important to consider that agents that interfere with chromatin modification states would also interfere with DNA methylation (Fig. 3). Razin and Cedar (1977) have shown two decades ago that methylated DNA is enriched in regions of the genome that are packaged in inactive chromatin configuration. The discovery of MBD and their ability to recruit HDACs provided a molecular mechanism for this relationship; however, the data suggested a unidirectional relationship whereby DNA methylation dictates an inactive chromatin structure (Nan *et al*., 1998). Indeed, cell culture experiments demonstrated that *in vitro* methylated plasmids were packaged in inactive chromatin following transfection into mammalian cells (Keshet *et al*., 1986). However, follow-up experiments demonstrated a bilateral relationship between

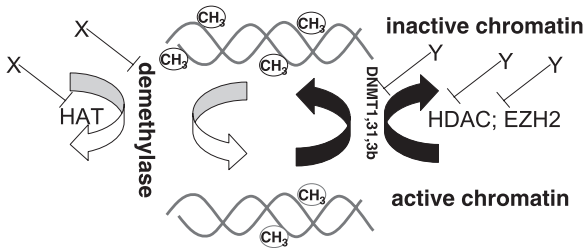

FIG. 3. The dynamic relationship between DNA methylation and chromatin structure, targets for toxic agents that alter DNA methylation patterns. The DNA methylation and chromatin modification equilibrium is laid down during embryogenesis. However, a balance of DNA methylation and demethylation activities as well as chromatin-activating modifications such as histone acetylation or inactivating modification such as histone deacetylation (catalyzed by HDACs) or H3K27 methylation (catalyzed by HMTase such as EZH2) dynamically maintains this pattern. The chromatin modification states and DNA methylation states are interrelated. Histone acetylation facilitates DNA demethylation, and histone H3K27 methylation facilitates DNA methylation. Therefore, agents that interact with the chromatin modification enzymes will also affect DNA methylation. Y-putative toxic agents that target DNMT1 or HDAC (such as TSA or valproic acid) will increase histone acetylation and facilitate DNA demethylation. X-putative toxic agents that target either the demethylase activities listed in Figure 2 or HAT will cause histone deacetylation and DNA hypermethylation.

chromatin modification and DNA methylation. The clearest example is the interrelationship between histone H3K27 methylation EZH2 methyltransferase and DNA methylation. Not only does EZH2 direct DNA methylation but also depletion of EZH2 can cause loss of methylation (Marker, 2007; Vire *et al*., 2006). This implies that agents that interact with HMTase interactors will affect DNA methylation in addition to histone methylation (Fig. 3).

Another well-documented connection between chromatin modification and DNA methylation is the relationship between histone acetylation and DNA methylation. Agents that inhibit HDACs could cause loss of DNA methylation. Sodium butyrate a general HDAC inhibitor was shown to trigger active DNA demethylation of EBV and cellular DNA in human cells (Szyf *et al*., 1985). The commonly used HDAC inhibitor *trichostatin A* (TSA) triggered loss of DNA methylation (Cervoni and Szyf, 2001; D'Alessio *et al*., 2007; Ou *et al*., 2007; Selker, 1998). Valproic acid is an excellent example of an agent that has been used for decades and was rediscovered as an HDAC inhibitor (Gottlicher *et al*., 2001). Several studies show that valproic acid could also cause DNA demethylation (Detich *et al*., 2003a; Milutinovic *et al*., 2007). Moreover, valproate causes DNA demethylation in the brain as well (Dong *et al*., 2005, 2008; Tremolizzo *et al*., 2002). Thus, in addition to the immediate pharmacological activity of valproic acid, it might leave a persistent mark in the methylome that would last well after the treatment and could have a latent and impact on health and behavior. Valproate is a good illustration of the cryptic DNA methylation toxicity of agents whose mechanism of action did not include initially DNA methylation. Although the effects of these agents on DNA methylation might be indirect, they impact the DNA methylation pattern through the interrelationship between DNA methylation and chromatin modification (Fig. 3).

DNA METHYLATION AND DISEASE: IMPLICATIONS FOR TOXICOLOGY

An important incentive to develop screens for agents that trigger DNA methylation modulation in standard safety tests is the increasing body of data suggesting involvement of DNA methylation in human disease. It is possible that several of these aberrations in DNA methylation associated with disease are introduced by adverse exposures and that by identifying and screening for agents that introduce changes in DNA methylation, it will be possible to reduce vulnerability to disease. Because changes in DNA methylation could be persistent, there could be a significant lag between exposure and appearance of disease. This creates a unique challenge for screening methodology and for establishing a causal relationship between the initial exposure and the disease. Aberrations in DNA methylation were reported in schizophrenia (Grayson *et al*., 2005; Petronis *et al*., 2003; Veldic *et al*., 2005), lupus

(Balada *et al*., 2007a,b, 2008; Yung and Richardson, 1994), and type II diabetes (Jiang *et al*., 2008; Junien and Nathanielsz, 2007; Kaminsky *et al*., 2006; Ling *et al*., 2008) and were proposed to participate in cardiovascular disease (Corwin, 2004; Gluckman *et al*., 2009a,b; Hanson and Gluckman, 2008).

Cancer has been the first disease to be proposed as a target for DNA methylation–targeted therapeutics (Szyf, 1994) and serves as a prototype for the toxic potential of agents targeting DNA methylation. Several types of aberration in the DNA methylation machinery occur in cancer: hypermethylation of tumor suppressor genes, aberrant expression of DNMT1 and other DNMTs, as well as hypomethylation of unique genes and repetitive sequences (Baylin *et al*., 2001; Ehrlich, 2002; Issa *et al*., 1993). Although hypermethylation of tumor suppressor genes has been the main focus of attention for the last decade, it is becoming clear that demethylation is equally important because critical genes for cancer growth and metastasis are demethylated in cancer (Pakneshan *et al*., 2004; Shteper *et al*., 2003; Shukeir *et al*., 2006). Several studies suggest that DNA demethylation plays an important role in turning on prometastatic genes such as *HEPARANASE* (Shteper *et al*., 2003), matrix metallopeptidse 2 (Shukeir *et al*., 2006), and *UROKINASE PLASMINOGEN ACTIVATOR* (Pakneshan *et al*., 2004).

SCREENING FOR DNA METHYLATION MODIFIERS

Screening chemicals for carcinogenicity traditionally focused on their mutagenic effects. Although it is evident that mutations could drive the cancer process, it is now well established that aberrations in DNA methylation play a critical and perhaps a more frequent role in cancer (Baylin and Herman, 2000). It is therefore critical to identify agents that could possibly modulate DNA methylation either directly or indirectly as potential carcinogens. There are certain examples of agents that alter DNA methylation and are associated with cancer. Ethanol exposure alters metabolism of SAM the universal methyl donor resulting in global hypomethylation, liver cirrhosis, and an increased risk for hepatocellular carcinoma (Purohit *et al*., 2007). Methyl-depleted diets were shown in rodents to increase the risk for hepatocellular carcinoma (Wilson *et al*., 1984) and induce hypomethylation of oncogenes (Zapisek *et al*., 1992). Inorganic arsenic, a human carcinogen, consumes SAM in its metabolism and causes global hypomethylation in epithelial liver cell cultures (Zhao *et al*., 1997). The relationship between SAM reduction and hypomethylation was questioned, however, in a later study that also showed sex differences in the response to arsenic but did not show a correlation between changes in SAM concentrations and global hypomethylation (Nohara *et al*., 2010). Cigarette smoke, which is indubitably the most established agent to trigger cancer, was shown to induce demethylation of a prometastatic oncogene *SYNUCLEIN-GAMMA* in lung cancer cells through downregulation of DNMT3B (Liu *et al*., 2007). These scattered but nevertheless striking examples point to the great need to develop a methodical and comprehensive evaluation of the DNA methylation alteration potential of agents as an integral part of assessing their safety. DNA methylation changes that are not as dramatic as those listed here might well result in a latent potential to develop cancer and other diseases. These less dramatic but nevertheless potentially significant alterations in DNA methylation go undetected by current carcinogenicity tests.

The potential long-term adverse effects of nongenotoxic compounds that alter DNA methylation force us to consider that any assessment of safety of drugs, foods, and dietary supplements as well as environmental assessments will involve a DNA methylation screen. Critical issues need to be addressed before such a screen is implemented by regulatory agencies: What model system should be used? What would be evaluated (gene specific, global changes, or genome-wide changes)? What model compounds might be evaluated? How should one select dose? What time points should be used? Should we be looking for transgenerational effects? As a policy, it might be advisable to screen all new agents for overt DNA methylation effects. This should be done in several phases. Although it might be difficult to detect all possible DNA methylation modifiers, especially those that have gene-specific or cell type–specific effects, it should be possible to identify DNA methylation modifiers that interfere with the basic DNA methylation/demethylation enzymes and interrelated factors such as chromatin-modifying enzymes. One possible approach is to use a DNA methylation reporter assay such as methylated Cytomegalovirus promoter-green fluorescence protein (GFP). We previously described HEK293 cells transiently transfected with either *in vitro* methylated or unmethylated promoter-GFP reporters to screen for agents that activate methylated genes or silence unmethylated genes (Cervoni and Szyf, 2001). Positive hits could then be validated for DNA methylation changes with pyrosequencing. This assay could be automated enabling screening of a large number of compounds using doses that are within the range of anticipated exposures in human populations. This assay was responsive in our hands to all known DNA methylation modifiers that we tested including those that acted directly on DNA methylating enzymes such as SAM (Detich *et al*., 2003b) and indirectly through other chromatin-modifying enzymes such as HDAC inhibitors (Cervoni and Szyf, 2001). Positive hits could also be further tested for their effects on global DNA methylation using global assays such as Luminometric Methylation Assay (Karimi *et al*., 2006). In the second phase, agents that scored positive could be tested in whole animals (rodents) and the immediate and long-term phenotypic impact will be determined in the first generation and transgenerationally. One elegant *in vivo* model for detecting DNA methylation modifier is the Agouti mouse model developed by Jirtle (Dolinoy *et al*., 2007b). Clearly, however, a caveat of these aforementioned assays is that they might miss agents that have gene-specific or tissue-specific effects. Nevertheless, these assays would detect a large number

of DNA methylation modifiers and test their potential long-term adverse effects. More comprehensive genome-wide methylation mapping and multiple tissue testing *in vivo* should be employed when there is additional data from animal experiments, clinical practice, or epidemiology that raise the suspicion of an epigenetic mechanism. Additional suspects that need to be studied comprehensively are analogs of known substrates of DNA methylation/demethylation and chromatin modification enzymes as well as analogs of known DNA methylation modifiers or other members of their classes of drugs.

IMPLICATIONS OF THE INVOLVEMENT OF DNA METHYLATION IN BEHAVIOR AND BEHAVIORAL DISORDERS ON DNA METHYLATION TOXICOLOGY

Recent advances in epigenetics of brain function and behavior have important implications on our understanding of DNA methylation–related toxicology. First, studies in brain behavior and memory were important in demonstrating that DNA methylation is dynamic in postmitotic cells. This obviously expanded the scope of toxicity of DNA methylation modifiers to nondividing cells as discussed above (Fig. 1). This also implies that agents that interfere with DNA methylation could have an impact on health and physiology at any point in life, and the effect must not be limited to dividing cells. Of specific concern is the possibility that agents that modify DNA methylation might have a lasting impact on behavior. This is highly relevant in the consideration of the safety of DNA methylation and HDAC inhibitors used in chemotherapy.

Second, studies on the association of DNA methylation and the long-term impacts of early-life adversity illustrate the life-long impact of DNA methylation alterations early in life. This should be of concern in assessing the safety of agents used in early life. Moreover, they illustrate how transient exposures can result in persistent changes in DNA methylation and phenotype. DNA methylation appears to be a mechanism for genomic memory of adverse exposures.

Third, studies in DNA methylation and behavior expand the scope of DNA methylation–associated toxicity. Adverse social environments could have a similar impact to adverse chemical environments on the epigenome. The boundary and classic chasm between toxic social and chemical exposure might be artificial, and perhaps, it is time to think about toxicity and toxicology from a broader point of view that include social, physical, and chemical exposures.

Fourth, studies in behavior have illustrated how signaling pathways are involved in translating a transient environmental signal into a persistent DNA methylation change. This again expands the scope of exposures that would result in DNA methylation toxicity. Interference with signaling pathways that control chromatin structure and DNA methylation would result in alterations in DNA methylation. Agents that act on signaling pathway might have lasting latent effects in addition to the immediate pharmacological impact of modulation of signaling pathways. The possibility of an indirect impact on DNA methylation that could have long-lasting implications must be considered when assessing the safety of agents whose pharmacological action on signaling pathway is seemingly well established and whose immediate effects on physiology and biochemistry are well documented.

The fifth lesson that will be discussed below is that the response of the methylome to an exposure might be highly organized rather than stochastic and therefore somewhat defined rather than chaotic.

The following are the pertinent examples from behavioral epigenetics that support the points listed above.

DNA METHYLATION MEDIATING THE LONG-TERM IMPACT OF EARLY-LIFE EXPOSURES TO DIFFERENT SOCIAL ENVIRONMENTS

Early childhood is very well known to be an important period for acquisition of behavioral phenotypes, and "nurture" has been known to be extremely critical in shaping these behavioral patterns. These processes are known to take place well after gestation during the early part of childhood and might extend even later in life. The critical long-standing question was what were the mechanisms that embedded the social environment in the biology of the infant in a way that remained stable for a lifetime. How did nurture affect "nature" by modulating the underlying innate genetically defined developmental program?

There are several models that measure the impact of early-life social environment on behavior and other health phenotypes later in life. Animal models could be used to test whether the impact of early-life social environment on the phenotype is mediated by genetic or epigenetic mechanisms. Maternal behavior plays a cardinal role in the behavioral development of mammals. Models of maternal deprivation in primates and rodents and natural variation in maternal care in rodents were used to demonstrate the profound impact of maternal care and nurture on a panel of phenotypes in the offspring that last into adulthood (Ruppenthal *et al.*, 1976). These models were also used to demonstrate that impact of nurture was independent of genetic predetermination. That is, the link between differential maternal care and differential offspring phenotype could not be explained as a consequence of inheritance of genetic differences from the mother. These studies provided the biological support for an epigenetic mechanism linking maternal care and the phenotype of the offspring.

In rodents, a model of natural variation in maternal care was originally used to study the impact of maternal care on stress behavior and stress responsivity in the offspring. In the rat, the adult offspring of mothers that exhibit increased levels of pup licking/grooming (LG) (i.e., high-LG mothers) over the first

week of life show increased hippocampal glucocorticoid receptor expression, enhanced glucocorticoid feedback sensitivity, decreased hypothalamic corticotrophin-releasing factor expression, and more modest hypothalamic-pituitary-adrenal axis stress responses compared with animals reared by low-LG mothers (Francis *et al*., 1999; Liu *et al*., 1997). A genetic explanation was ruled out by cross-fostering studies. If indeed the maternal care behavior and stress responsivity in the offspring are genetically linked by an inherited polymorphism, then this phenotype of stress responsivity could only be transferred through the biological mother. However, cross-fostering experiments showed that the fostering mother and not the biological mother behavior defined the stress responsivity of the offspring that lasted into adulthood excluding a genetic explanation (Francis *et al*., 1999; Liu *et al*., 1997). We therefore tested the possibility that maternal behavior resulted in differential DNA methylation in the offspring hippocampus. Our original approach focused on a candidate gene examining a gene known to be involved in regulation of stress responsivity through feedback inhibition, the gene encoding the glucocorticoid receptor. Differences in DNA methylation and histone acetylation in the regulatory regions of the glucocorticoid receptor (GR exon 1_7 promoter) gene were observed in the hippocampus of the offspring of high- and low-LG mothers. Differences in epigenetic programming in response to differences in maternal LG emerged early in life and remained stable into adulthood (Weaver *et al*., 2004). A limitation of this early study was that the data do not tell us if changes are occurring in neurons, astrocytes, and glia or in two or all three of these cell types.

The basic concepts of this study were repeated more recently in several other models of early-life social adversity. Exposure of infant rats to stressed caretakers that displayed abusive behavior produced persisting changes in methylation of brain-derived neurotrophic factor (*BDNF*) gene promoter in the adult prefrontal cortex (Roth *et al*., 2009). Early-life stress (ELS) in mice caused sustained DNA hypomethylation of an important regulatory region of the *arginine vasopressin* (*AVP*) gene (Murgatroyd *et al*., 2009).

Both adverse chemical and social exposures at this early period in life could have a long-lasting impact on behavior and memory through changes in DNA methylation. DNA methylation toxicology should be extremely careful in uncovering potential DNA methylation modifiers acting at this time point in life.

SIGNALING CASCADES LEADING FROM MATERNAL CARE TO EPIGENETIC PROGRAMMING

An important question that remains to be answered is what are the mechanisms that link exposure to a social experience and differential DNA methylation in the hippocampus. These have broad implications on our understanding of the scope of exposures that could affect DNA methylation. DNA methylation could therefore be affected not only by agents that directly interact with the DNA methylation machinery but also with agents that trigger responses such as the response to social adversity. It is clear that in the case of social adversity, the exposure does not directly interact with the DNA methylation machinery. A reasonable hypothesis is that exposure to social cues results in firing of a signaling cascade that activates transacting factors that deliver DNA and chromatin-modifying enzymes to specific regulatory sequences of genes. Evidence for such a mechanism comes from the rat maternal care model. Maternal behavior triggers a signaling pathway that involves the serotonin receptor, increase in cAMP, recruitment of the transcription factor NGFI-A, which in turn recruits the histone acetyltransferase (HAT) CBP, and the MBD and candidate DNA demethylase MBD2 (Weaver *et al*., 2007) to the *GR* promoter. Our hypothesis is that the increased histone acetylation triggered by CBP or by other recruited HAT facilitates the demethylation of the gene by MBD2 or other DNA demethylases (unpublished data). It is yet unknown whether any of the activities implicated in DNA demethylation discussed above (Fig. 2) are present in the brain or triggered by maternal care. For example, 5-hydroxymethylcytosine was proposed to be an intermediary of the DNA demethylation reaction (Hamm *et al*., 2008) and was originally discovered in the brain (Kriaucionis and Heintz, 2009). TET1 catalyzes the hydroxylation of 5-methylcytsoine and was shown to be required for DNA demethylation in embryonal stem cells (Ito *et al*., 2010). It is tempting to speculate that it participates in demethylation in the hippocampus, but this remains to be examined by future experiments.

These data chart a possible conduit through which exposure to a social behavior such as maternal behavior results in epigenetic modification of a specific gene in the brain. Although it is certain that there are other molecular pathways that link social exposure and changes in DNA methylation in particular positions in the genome, this example provides evidence for the feasibility of transducing a social signal into a DNA methylation mark. A similar response might be triggered to a chemical exposure resulting in long-term DNA methylation–mediated toxicity.

A different signaling cascade linking social exposure to DNA demethylation was proposed more recently to explain how ELS results in persistent life-long hypomethylation of the *AV*P gene. *AVP* promoter is methylated and bound by the MBD MeCP2. Depolarization of hypothalamic neurons triggers phosphorylation of MeCP2 at Ser438 by calcium-dependent calmodulin kinase II (Murgatroyd *et al*., 2009). This phosphorylation converts MeCP2 from a transcriptional silencer with high affinity to methylated DNA into a transcriptional activator with low affinity to methylated DNA (Zhou *et al*., 2006). This facilitates demethylation of the *AVP* gene. The change in MeCP2 affinity to the methylated DNA by phosphorylation in response to neuronal activation was shown before to facilitate demethylation of the BDNF promoter (Chen

et al., 2003). This signaling pathway delineates a direct link between neuronal activation and the phosphorylation state of a protein interacting with methylated genes in the brain. Neuronal activation resulting in signaling through phosphorylation of proteins interacting with methylated DNA might be a general pathway that links social exposure and the activation of neurons. Agents affecting these signaling cascades could result in changes in DNA methylation. Some of these changes would possibly remain as persistent memories of the transient activation of the signaling pathway.

REVERSIBILITY OF EPIGENETIC PROGRAMMING BY MATERNAL CARE

The changes in DNA methylation in response to ELS remain into adulthood, and they could possibly explain how early-life experience would shape the phenotype. However, because DNA methylation is potentially dynamic as discussed above, the critical question is whether patterns of epigenetic modification programmed early in life are final states or whether they are potentially reversible. The idea that epigenetic states might be reversible even in adult brain as well as other tissues has immense implications on the potential for interventions to override the effects of early-life adversity as well as for toxicology. If indeed DNA methylation patterns are reversible, then exposures later in life might continue and reset the epigenome including the brain. Moreover, DNA methylation might participate in ongoing physiological processes in the brain that require reprogramming of gene expression such as learning and memory. Toxic agents that interfere with these processes and alter DNA methylation could have a range of effects acting at different timescales from disrupting memory and physiological responses to reversing long-term early-life adaptive epigenetic programming.

Our previous studies have shown that increasing histone acetylation using the HDAC inhibitor TSA facilitates replication-independent demethylation of nonreplicating plasmid DNA in human cells (Cervoni and Szyf, 2001; Cervoni *et al*., 2002). This experiment demonstrated that human somatic cells contain the enzymes required to demethylate DNA in the absence of DNA replication and that it is possible to alter the DNA methylation pattern using pharmacological agents that change histone acetylation. We tested whether a similar strategy would reverse epigenetic states established through maternal care and whether they would result in a change in the phenotype. Injection of the HDAC inhibitor TSA into the brains of adult offspring of low-LG maternal care reversed the epigenetic programming of the GR exon 1_7 promoter and reestablished stress responsivity and open-field behavior that was indistinguishable from the offspring of high-LG maternal care (Cervoni and Szyf, 2001; Cervoni *et al*., 2002).

We have previously shown that it is possible to alter the state of methylation of a nonreplicating plasmid in the opposite way by treating cells with the methyl donor SAM, which inhibits the DNA demethylation reaction (Detich *et al*., 2003b.). Injection of the amino acid methionine, the precursor of SAM, into the brains of adult offspring of high maternal LG resulted in increased DNA methylation and downregulation of *GR* as well as heightened stress responsivity and an open-field behavior that was indistinguishable from the adult offspring of low maternal LG (Weaver *et al*., 2004, 2005). These data suggest that both the methylating and demethylating enzymes are present in the adult neuron and are amenable to pharmacological modulation. Similarly, they are potentially vulnerable to toxic exposures.

DYNAMIC DNA METHYLATION PATTERNS AND MEMORY ACQUISITION

Recent studies that tested the involvement of DNA methylation in memory acquisition using a "fear conditioning" model demonstrated that both methylation and demethylation activities are present in mature neurons as predicted by Weaver *et al*. (2004, 2005) and that they are involved in memory acquisition through rapid methylation and transcriptional silencing of the memory suppressor gene *PP1* and demethylation and transcriptional activation of the synaptic plasticity gene *reelin* (Miller and Sweatt, 2007; Miller *et al*., 2008). These data demonstrate a new role for DNA methylation in the functional physiology of the brain in addition to its role in cellular differentiation and genome adaptation early in life. It also supports the proposition that DNA methylation participates in genome function at different levels and at different timescales. An open question is whether the participation of DNA methylation in memory is limited to regulation of gene expression or that it encodes memory through other genomic functions and genomic structures.

Evidence that the DNA methylation pattern is dynamic in hippocampal neurons and that it is maintained by a dynamic balance between methylase and demethylase activities was provided by showing that blocking one side of the DNA methylation equilibrium by conditional knockout of *dnmt1* and *dnmt3a* methyltransferases in adult neurons resulted in loss of DNA methylation in the absence of cell division (Feng *et al*., 2010). Combined, these lines of evidence discussed here support the idea that although epigenetic programs established early in life could persist throughout life, they are maintained by a dynamic equilibrium of DNA methylation and demethylation and are potentially changeable by the appropriate interventions. Our data provide an example for how pharmacological intervention might do this. Agents that interact with the DNA methylation machinery could therefore affect DNA methylation pattern in somatic cells. A provocative possibility and concern is that epigenetic programs established early in life could be adversely reversed in adults not only by chemical agents but also by social and cognitive exposures.

EPIGENETIC PROGRAMMING BY EARLY-LIFE EVENTS IN HUMANS: PERSISTENT LONG-TERM IMPACT OF ADVERSE EXPOSURES ON DNA METHYLATION AND THE PHENOTYPE

A critical question for discussion of the possible implications of DNA methylation "toxins" is whether the results in rodents could be translated to humans. We tested whether there was any evidence that early childhood adversity was associated with epigenetic marks later in life. If this is indeed true, it might provide an explanation for the accumulating evidence of a link between early-life experience and behavior and health later in life. DNA methylation patterns are tissue specific as discussed above, and the brain is inaccessible to such studies in living subjects. We therefore took advantage of a well-phenotyped brain bank to address the question of whether early-life adversity leaves its mark in the DNA methylation pattern of the adult human brain. We first focused on the hippocampus and examined a cohort of suicide victims in Quebec who were abused as children and their control group. The first study looked at the promoters of the rRNA genes. rRNA forms the skeleton of the ribosome, the protein synthesis machinery. Protein synthesis is essential for building new memories and creating new synapses in the brain. Our genome contains around 400 copies of the genes encoding rRNA. One possible way to control the protein synthesis capacity of a cell is through changing the fraction of active rRNA alleles in a cell by DNA methylation of a subset of these alleles (Brown and Szyf, 2007). Our results showed that the suicide victims who experienced childhood abuse had higher overall methylation in their rRNA genes and expressed less rRNA (McGowan *et al*., 2008). In our second study, we examined whether epigenetic differences were driven by early childhood adversity or by other processes leading to suicide. We compared suicide completers who were abused as children to suicide completers who were not. This time we looked at the GR exon 1f promoter that is homologous to the promoter affected by maternal care in the rat. Site-specific differences in DNA methylation in the GR exon 1f promoter between suicide completers who had reported social adversity early in life and suicide completers who did not experience social adversity early in life were detected (McGowan *et al*., 2009). These data are a first demonstration that it is possible to identify the epigenetic imprints of early-life exposure in the epigenome in the adult brain. They also illustrate the possible long-term and latent impact of DNA methylation changes in the brain that were introduced in response to adverse exposures. This creates a unique challenge for toxicology, how to screen and test agents that precipitate persistent long-lasting changes in DNA methylation in the brain and possibly other somatic tissues.

Alcohol is a classic example of an agent that has long-term toxic impacts especially during gestation and could serve as a model for studying potential "toxicomethylomics." Chronic ethanol exposure during gestation and lactation affects SAM levels in the liver of the offspring in rats (Murillo-Fuentes *et al*., 2005). Chronic ethanol consumption of pregnant mice leads to demethylation of fetal DNA (Garro *et al*., 1991). Chronic ethanol exposure of fetal cortical neurons from mice results in demethylation of the glutamate receptor *N*-Methyl-D-aspartic acid subtype 2B (*NR2B*) (Marutha Ravindran and Ticku, 2004), whereas chronic intermittent alcohol treatment of primary cortical neurons induces site-specific demethylation and induction of expression of the *NR2B* gene (Qiang *et al*., 2010). A study in human peripheral blood cells in alcohol users suggests inverse correlation between DNA methylation of *NR2B* and severity of drinking pattern (Biermann *et al*., 2009), supporting the hypothesis that DNA methylation alterations in *NR2B* play a role in the long-term impacts of alcohol consumption on *NR2B* expression and behavior. A study of global DNA methylation in alcoholism subjects has shown, however, overall increase in genomic methylation that was surprisingly associated with reduction in DNMT3B expression (Bonsch *et al*., 2006). Although the impact that alcohol has on DNA methylation is well established, the mechanisms leading to changes in DNA methylation following alcohol exposure are unclear. The possible role of reduced SAM levels by ethanol has been proposed, but another study has shown that alcohol inhibition of methionine synthesis in the rat is compensated by an adaptive increase in betaine-homocysteine methyltransferase activity (Barak *et al*., 1987). A different mechanism for ethanol action is interfering with either the activity or expression of DNA methylation enzymes (Garro *et al*., 1992).

GENOME-WIDE AND SYSTEM-WIDE EFFECTS OF EARLY-LIFE ADVERSITY

The first studies examining DNA methylation in the brain described above focused on a few gene suspects that were very well known to mediate stress behavior as well as other dedicated brain functions. Most of our studies were biased toward the candidate gene approach assuming that phenotype associated with early-life adversity would involve a few critical brain-specific genes. In addition, unsurprisingly, the first line of studies examining the impact of early-life adversity focused on specific brain regions. However, it is becoming clear that genes work in networks and that the total output of a network could be significantly affected by a combination of subtle changes in several nodes of a network. We recently performed a detailed mapping of five megabases of DNA spanning the locus of the GR gene from both directions and identified numerous differences in DNA methylation between the suicide and the control groups. Recent high-density epigenome mapping of chromosome 18 in the adult rat offspring of high and low maternal care reveals broad differences in DNA methylation and histone acetylation that cover wide regions of chromosome 18 (McGowan *et al*., 2011). High maternal care results in hypomethylation of some regions and hypermethylation of

others, and an inverse picture is observed with histone acetylation. This can explain why the adult offspring of high- and low-LG mother exhibit widespread differences in gene expression (Weaver *et al.*, 2006). These data have immediate implications on studying, screening, and testing the potential adverse impact of suspect agents. Whole-methylome approaches might be necessary to evaluate the potential toxicity of candidate agents. The Yellow agouti (A(vy)) mice described above could serve as a model for screening agents that alter DNA methylation during gestation. This assay provides a remarkably simple and attractive coat color detection (Dolinoy *et al.*, 2006; Waterland and Jirtle, 2003). However, it is unclear whether this assay would detect only a subset of DNA methylation effects such as those involved in repetitive transposons, which regulate the *agouti* gene, and it might not detect other kinds of DNA methylation modulations during gestations. It is essential to therefore develop and identify alternative assays. Another intriguing possibility is developing behavioral models such as stress responsivity in adults for screening DNA methylation modulators during early life. Alternatively, rather than using phenotypic screens for DNA modulators, it might be possible to examine the effect of the reagents on the methylome in cell culture followed by *in vivo* tests. Perhaps, a combination of human cell lines and several animal models would provide a reasonable coverage of the main suspects.

SUMMARY AND PROSPECT

DNA methylation is changing the way we approach toxicity by expanding the temporal lag between exposure and phenotype, DNA methylation acting as a persistent memory in the genome of an environmental exposure. An outstanding complexity is the possibility that DNA methylation–modifying agents will leave their mark not only in one generation but also across generations (Anway *et al.*, 2005). The concept of transgenerational transmission of DNA methylation alterations to future generations in response to exposure in the first generation is gaining further experimental support with the demonstration of specific exposure-induced transgenerational sperm methylation pattern (Guerrero-Bosagna *et al.*, 2010). A recent provocative study has shown that ELS affects a depressive phenotype that is transmitted through generations through the male germ line and is associated with changes in DNA methylation in sperm and the brain (Franklin and Mansuy, 2010).

Our increased understanding of the role of DNA methylation in mediating long-term effects of adverse exposures introduces new complexity to the task of the assessment of safety of chemicals and is bound to change the way we call certain agents safe. DNA methylation could serve as a long-term memory of exposures to toxic agents. Agents that block DNA methylation or demethylation activities could alter DNA methylation patterns and have a latent effect that could express itself phenotypically as a disease or health challenge long after the exposure is gone. Especially critical are exposures during the perinatal period. This poses a great challenge for safety assessment of agents. Several assays were proposed here to detect DNA methylation–modifying agents, but the question remains of how do we identify agents that affect DNA methylation of certain genes and in certain tissues only. One important question is how do we determine whether the numerous agents that are considered safe and approved by regulatory agencies and are extensively used in the population are DNA methylation modifiers. Which of our common environmental exposures is acting on DNA methylation? Which of these exposures should we test?

DNA methylation expands the scope of toxicology by offering a common platform to a range of toxic exposures from the physical environment to the social environment. DNA methylation points to the notion that toxicity is not limited to the physical and chemical spheres but could include even toxic social encounters (Szyf *et al.*, 2008).

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